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THE EFFECT OF PHENOBARBITONE PRE-TREATMENT ON VITAMIN K_1 DISPOSITION IN THE RAT AND RABBIT

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Abstract—The effect of phenobarbitone enzyme induction on the pharmacokinetics of an intravenous pharmacological dose (1 mg/kg) of vitamin K_1 was studied in the rabbit. Phenobarbitone pretreatment significantly ($P < 0.01$) increased the plasma clearance of vitamin K_1 and decreased the terminal (β) half-life from 2.08 ± 0.56* to 0.99 ± 0.30 hr. However, phenobarbitone pretreatment did not alter the pharmacodynamic response to vitamin K_1, measured as the increase in prothrombin complex activity, in brodifacoum-anticoagulated rabbits. In the rat, phenobarbitone enzyme induction increased the extent and rate of biliary excretion of polar vitamin K_1 metabolites following intravenous administration of the vitamin. Perturbation of vitamin K_1 metabolism by phenobarbitone enzyme induction is not dependent on the concentration of the vitamin. The greater hepatic elimination resulted in lower systemic blood concentrations of both vitamin K_1 and the 2,3-epoxide. A similar reduction in the concentration of vitamin K_1 in the blood of epileptic mothers treated with anticonvulsants such as phenobarbitone may explain the coagulation defect frequently observed in their offspring [K. R. Mountain, J. Hirsh and A. S. Gallus, *Lancet* ii, 265 (1970)].

Neonates born to epileptics on long-term anticonvulsant therapy, including phenobarbitone or phenytoin, often have a coagulation defect at birth [1]. The coagulation defect is not found in the mothers of the affected neonates and it appears to be similar to the defect observed in vitamin K_1 deficiency. Thus there is a decrease in the activity of vitamin K_1-dependent clotting factors II, VII, IX and X which can be corrected by vitamin K_1 administration. In contrast, the activity of vitamin K_1-independent clotting factors V, VIII and fibrinogen is normal.

Chronic enzyme induction may enhance the metabolic inactivation of a number of endogenous compounds [2]. For example, rifampicin, antipyrine and phenobarbitone increase the rate of vitamin D metabolism, although physiological disturbances are observed only after long-term treatment or in patients with malnutrition [3]. In this paper we report the effects of phenobarbitone enzyme induction on the disposition of vitamin K_1 in the rat and rabbit. The rabbit was chosen for pharmacokinetic studies as it is a suitable animal model for investigating the plasma metabolites of vitamin K_1 [4], while the rat was used to study the hepatic disposition and biliary excretion of vitamin K_1 because of its suitability for such experimental procedures [5].

MATERIALS AND METHODS

Animals. Male New Zealand White rabbits (2.5–3.0 kg) were given either phenobarbitone (20 mg/kg twice daily for 4 days) or saline (2 ml/kg daily for 4 days) intraperitoneally (i.p.). The animals were kept in cages with wire mesh floors with free access to water and food (Diet R14 Labsure Animal Foods, Poole, U.K.) and left for 18 hr after the last injection of either phenobarbitone or saline prior to vitamin K_1 administration. Vitamin K_1 (phylloquinone, Konakion, Hoffman La Roche, Basel, Switzerland) was given intravenously into the marginal ear vein and 4 ml blood samples were collected from the opposite ear vein at 0.25, 0.5, 0.75, 1, 2, 3, 4, 5 and 6 hr. Plasma was removed after centrifugation (8000 *g* for 10 min) and stored at −20° until required for assay purposes.

In order to determine the effect of phenobarbitone enzyme induction on the pharmacological response to vitamin K_1, two groups of rabbits were given either phenobarbitone or saline as described above. Immediately after the final injection, brodifacoum (10 mg/kg) in polyethylene glycol (0.1 ml/kg) was administered intravenously to each group of animals. The response to vitamin K_1 (1 mg/kg; Konakion), given intravenously 20 hr later, was monitored in both groups of animals by measuring plasma prothrombin complex activity at regular intervals thereafter.

Male Wistar rats (200–250 g, Bantin and Kingman, Hull, U.K.) were given phenobarbitone (40 mg/kg twice daily for 5 days) or saline (2 ml/kg for 5 days) by i.p. injection. Animals were given free access to food and water. Eighteen hours following the last injection the rats were anaesthetized with 15% urethane (2 ml/kg i.p.) and tracheaostomized with polypropylene tubing (PP250). PP50 cannula was inserted into the left superficial jugular vein for the intravenous administration of vitamin K_1. The common bile duct was cannulated with PP25 tubing. Body temperature was maintained during the experiment by heat lamps and was monitored using a rectal probe thermistor. Following completion of the dissection procedure, the animals were left to stabilize

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for at least 15 min. Groups of control and enzyme-induced rats were given [^{3}H]phylloquinone (20 μCi/kg; 0.45 μg/kg: Hoffman La Roche, Basel, Switzerland) with or without a simultaneous pharmacological dose of 1 mg/kg vitamin K_1 (Konakion). Bile was collected into weighed microcentrifuge tubes at 30 min intervals for 5 hr. At the end of this period, a blood sample was taken by cardiac puncture, centrifuged at 10,000 *g* for 2 min and stored at −20°. The liver was removed, blotted dry, weighed and immediately frozen to −20° and stored until required for assay purposes.

Analysis of vitamin K_1 and vitamin K_1 2,3-epoxide in rabbit plasma. High-performance liquid chromatography (HPLC) was carried out using the following components: an Altex 110A isocratic solvent delivery pump, an Altex 160 fixed wavelength UV detector connected to a Gilson N1 potentiometric recorder. Normal-phase HPLC was used with a mobile phase of 0.2% acetonitrile in hexane pumped at 2 ml/min (500–1500 psi). UV detection was effected at 254 nm with a sensitivity of up to 0.002 aufs. The normal-phase system used a Partisil-10 column (25 cm × 4.5 nm i.d., 10 μm particle diameter, Whatman) protected by a guard column (2.5 cm × 4.5 mm i.d.) packed with Partisil-10 silica gel. Column efficiency was typically greater than 20,000 plates/m for all test compounds including the internal standard.

Chemicals and reagents were of analytical grade. All solvents were HPLC grade (Rathbone Chemicals Ltd., Walkerburn, Scotland, U.K.). Vitamin K_1 2,3-epoxide was synthesized by the method of Tishler *et al.* [6], the structure confirmed by UV absorbance between 200 and 400 nm, and its purity verified by normal-phase HPLC. No residual vitamin K_1 was detected. The internal standard used was vitamin MK4 (2-methyl-3-farnesyl farnesyl-1,4-naphthoquinone) and was a gift from Hoffman La Roche (Basel, Switzerland). Standard solutions of 5, 50 and 500 μg/ml of vitamin K_1, its 2,3-epoxide and MK4 were prepared in hexane and stored protected from fluorescent light. These solutions were used to construct calibration curves calculated from the peak height ratios of vitamin MK4 to either vitamin K_1 or vitamin K_1 2,3-epoxide at concentrations over the pharmacological range observed in rabbit plasma. Plasma recoveries were greater than 90% for all standards at a concentration of 0.2 μg/ml, and plasma analysis was in the range of 0.02–10 μg/ml giving a limit of detection of 20 ng/ml. Linear regression lines were obtained from the standard graphs and were $y = 0.3597x + 0.0101$, $r = 0.994$ for *cis*-vitamin K_1, $y = 0.8939x + 0.0185$, $r = 0.993$ for *trans*-vitamin K_1 and $y = 0.4180x + 0.0289$, $r = 0.995$ for vitamin K_1 2,3-epoxide. Intra-assay variation, calculated from repeated sampling of a single spiked plasma sample, gave a coefficient of variation of 3.6%. The coefficient of variation of the slopes of the standard graphs, calculated over a 2 month period, was 7.0%. Glassware was first rinsed with 5% dimethyldichlorosilane in toluene and thereafter methanol-rinsed. The assay procedure involved taking an aliquot of the internal standard solution in a glass tube and blowing dry under nitrogen. Plasma (1 ml) was added, the tube vortexed for 30 sec and left at room temperature to equilibrate for 15 min. Following equilibration of the plasma, an equal volume of methanol was added, the tube shaken mechanically for 2 min, then a further 5 ml hexane was added and the tube shaken again for 5 min. To ensure complete separation of the methanol–water phase from the hexane layer, the tube was centrifuged for 1 min at 500 *g*. The hexane layer was removed, evaporated under nitrogen and redissolved in 100 μl of eluant. A 20 μl sample of this solution was injected into the chromatograph.

Analysis of [^{3}H]*vitamin K_1 and* [^{3}H]*vitamin K_1 2,3-epoxide in rat bile and plasma.* Thin layer chromatography (TLC) was performed on ether–acetone (4:1) extracts of liver homogenates as previously described [5].

Measurement of prothrombin complex activity (PCA). Blood samples (0.9 ml) were collected into 3.8% trisodium citrate (0.1 ml) and PCA measured as described previously [4].

RESULTS

The phenobarbitone dosing schedule designed to induce the hepatic mixed function oxidase system is well documented for the rat. Administration of phenobarbitone (80 mg/kg) for 5 days gives a 2-fold increase of both hepatic cytochrome P-450 and hepatic cytochrome *c* reductase and a decrease in pentobarbitone sleeping time [7]. For the rabbit, a dose of 40 mg/kg was given, which is close to the maximum tolerable for this species. Pentobarbitone

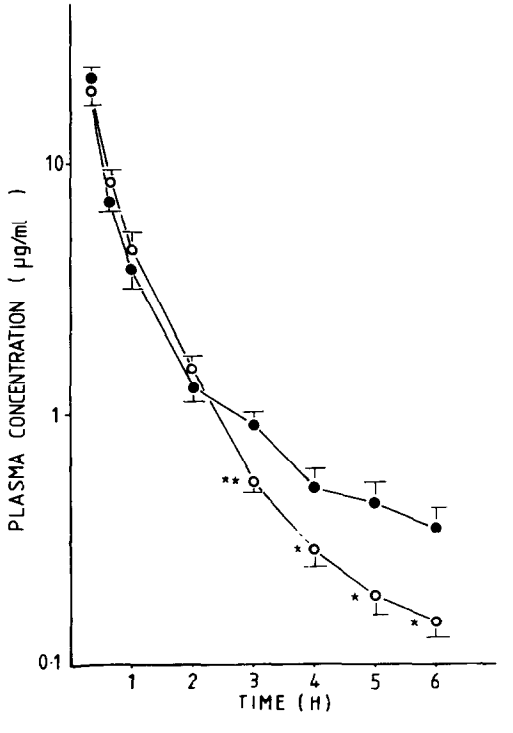

Fig. 1. Plasma concentrations of vitamin K_1 in control rabbits (●) and in phenobarbitone-pretreated rabbits (○) at various times after i.v. administration of vitamin K_1 (1 mg/kg). The results are expressed as means ($n = 4$) ± S.D. * $P \leq 0.05$; ** $P \leq 0.01$ (Student's *t*-test).

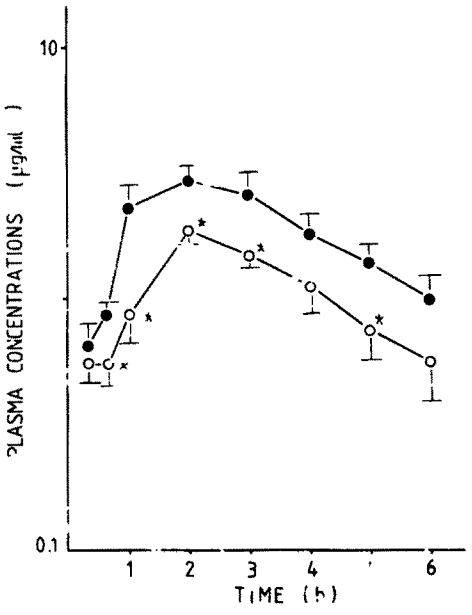

Fig. 2. Plasma concentrations of vitamin K_1 2,3-epoxide in control rabbits (●) and in rabbits pretreated with phenobarbitone (○) at various times after i.v. administration of vitamin K_1 (1 mg/kg). The results are expressed as means ($n = 4$) ± S.D. * $P \leq 0.05$ (Student's t-test).

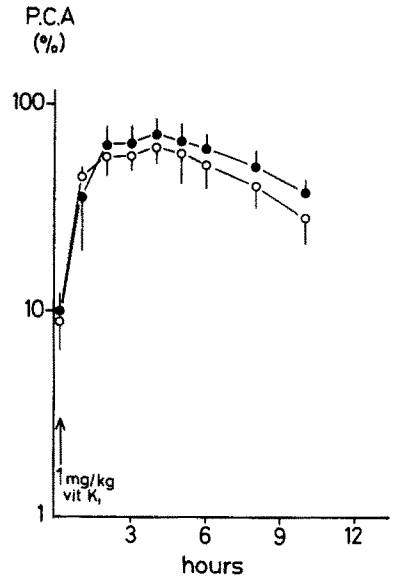

Fig. 3. Prothrombin complex activity (PCA) for control rabbits (○) and for phenobarbitone-pretreated rabbits (●) at various times after i.v. administration of vitamin K_1 (1 mg/kg). Animals were anticoagulated 20 hr prior to vitamin K_1 administration with brodifacoum (10 mg/kg, i.p.).

sleeping time was determined in groups of four rabbits as the time between loss and recovery of the righting reflex after i.p. injection of 40 mg/kg pentobarbitone. Control sleeping time was 115 ± 7.6 min ($n = 4$; mean ± S.E.M.) and this was decreased significantly ($P < 0.01$) after phenobarbitone treatment to 47.5 ± 15.9 min.

Effect of phenobarbitone on vitamin K_1 and vitamin K_1 2,3-epoxide pharmacokinetics in rabbit plasma

The effect of phenobarbitone pretreatment on the plasma levels of vitamin K_1 and its 2,3-epoxide following 1 mg/kg vitamin K_1 is shown in Figs. 1 and 2. The plasma concentration–time curves obtained for the vitamin and its 2,3-epoxide were similar to those previously obtained after a tracer dose in man [8, 9], the rabbit [10] and the rat [11]. The pharmacokinetic parameters are shown in Table 1. Phenobarbitone significantly ($P \leq 0.01$) decreased the slow (B) half-life of the plasma concentration-time curve for vitamin K_1 from 2.08 ± 0.56 to 0.99 ± 0.30 hr. Phenobarbitone did not, however, affect the half-life of vitamin K_1 2,3-epoxide. The plasma clearance of vitamin K_1 was increased from 0.61 ± 0.02 ml/min in the control group to 0.81 ± 0.14 ml/min in the phenobarbitone-induced animals.

Effect of phenobarbitone on the pharmacodynamic response of vitamin K_1 in brodifacoum anticoagulated rabbits

Figure 3 shows the change in PCA in control and phenobarbitone-induced anticoagulated rabbits fol-

Table 1. The effect of phenobarbitone enzyme induction in the rabbit on the pharmacokinetics of an intravenous dose (1 mg/kg) of vitamin K_1

	Control	Phenobarbitone
Vitamin K_1 $t_{\frac{1}{2}}\alpha$(h)	0.18 ± 0.01	0.14 ± 0.02
Vitamin K_1 $t_{\frac{1}{2}}\beta$(h)	2.08 ± 0.56	0.99 ± 0.30**
Vitamin K_1 A.U.C. (μg/ml h)	27.08 ± 1.08	20.88 ± 3.60*
Vitamin K_1 V.D. (L/kg)	0.098 ± 0.006	0.068 ± 0.018*
Plasma clearance (ml/min/kg)	0.609 ± 0.023	0.807 ± 0.142*

Values are expressed as means ($n = 4$) ± S.D. Student's unpaired t-test for significance: * $P \leq 0.05$; * $P \leq 0.01$. Pharmacokinetic parameters defined assuming a two-compartment model: A.U.C. = area under the plasma concentration–time curve; V.D. = apparent value of distribution = $\frac{\text{dose}}{\text{A.U.C.} \times \beta}$; plasma clearance = V.D. × β.

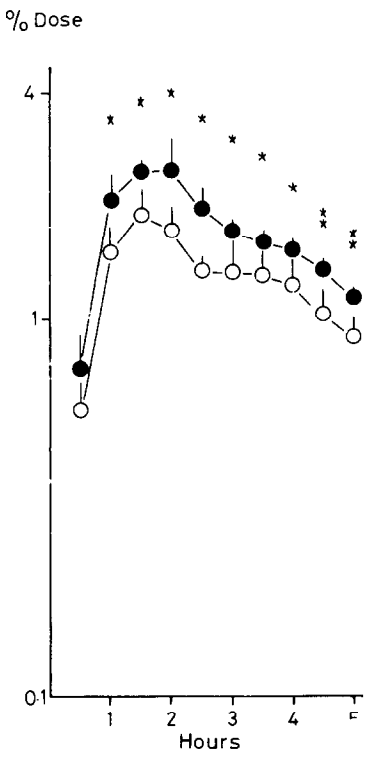

Fig. 4. Biliary excretion of radiolabelled metabolites of vitamin K_1 in the rat as percentage of administered dose following i.v. administration of [^{3}H]vitamin K_1 (20 μCi/kg; 0.45 μg/kg). Saline control animals (○); phenobarbitone-pretreated animals (●). Values are expressed as means ($n = 6$) ± S.D. * $P \leq 0.05$; ** $P \leq 0.01$ (Student's t-test).

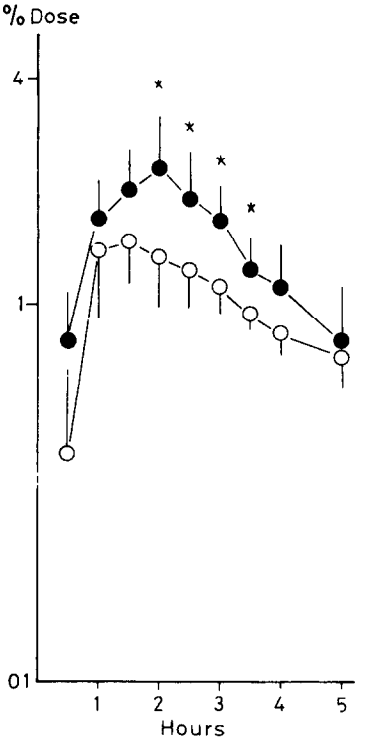

Fig. 5. Biliary excretion of radiolabelled vitamin K_1 metabolites in the rat as percentage of administered dose following i.v. dosing with [^{3}H]vitamin K_1 (20 μCi/kg; 0.45 μg/kg) and vitamin K_1 (1 mg/kg). Saline control animals (○), phenobarbitone-pretreated animals (●). Values are expressed as means ($n = 5$) ± S.D. * $P \leq 0.05$ (Student's t-test).

lowing 1 mg/kg vitamin K_1. Chronic enzyme induction did not alter the degree or duration of the response to vitamin K_1 in brodifacoum anticoagulated rabbits.

Effect of phenobarbitone on the biliary excretion of [^{3}H]vitamin K_1 and metabolites in rat bile

The phenobarbitone dosing regime produced an increase in bile flow in both induced groups. Cumulative bile flow up to 5 hr after a tracer dose of vitamin K_1 was significantly ($P < 0.01$) increased from 3.2 ± 0.7 g in control animals to 3.9 ± 0.2 g in the induced group. The 5 hr cumulative bile weights in control and phenobarbitone-treated rats that received a pharmacological dose of vitamin K_1 were 3.8 ± 0.6 and 4.7 ± 0.6 g, respectively.

Table 2. Hepatic concentrations of [^{3}H]vitamin K_1 [^{3}H]vitamin K_1 2,3-epoxide and total radioactivity 5 hr after administration of [^{3}H]vitamin K_1 (20 μCi/kg; 0.45 μg/kg or 1 mg/kg)

	Total ^{3}H (% dose)	Vitamin K_1 (% dose)	Vitamin K_1 2,3-epoxide (% dose)	Vitamin K_1 2.3-epoxide / Vitamin K_1
A. Tracer dose (0.45 μg/kg)				
Control ($n = 5$)	31.5 ± 6.6	14.2 ± 2.5	1.06 ± 0.54	0.087 ± 0.035
Phenobarbitone ($n = 6$)	32.4 ± 4.0	16.1 ± 2.0	1.48 ± 0.37	0.092 ± 0.018
B. Pharmacological dose (1 mg/kg)				
Control ($n = 4$)	40.0 ± 11.5	21.0 ± 5.9	2.40 ± 0.49	0.122 ± 0.040
Phenobarbitone ($n = 5$)	49.6 ± 16.9	24.9 ± 8.2	2.44 ± 0.68	0.105 ± 0.031

Hepatic concentrations of radioactivity, [^{3}H]vitamin K_1 and vitamin K_1 2,3-epoxide in the rat are expressed as a percentage of the administered dose. Values are expressed as means ± S.D.

The rate of biliary excretion of [^{3}H]vitamin K_1 metabolites over 5 hr after administration of a tracer dose of the vitamin to rats is shown in Fig. 4. Phenobarbitone increased the excretion rate of radiolabelled products which became apparent and significant ($P \leq 0.05$) 1 hr after radiolabelled vitamin K_1 was administered. The corresponding data obtained from control and phenobarbitone-induced rats given a pharmacological dose (1 mg/kg) of vitamin K_1 along with the radiolabelled tracer are shown in Fig. 5. The profile of excretion of radiolabelled material was similar in both experimental groups with phenobarbitone increasing significantly ($P \leq 0.05$) the rate of appearance of [^{3}H]vitamin K_1 metabolites in bile in the rat. TLC analysis of liver and plasma concentrations of radioactivity revealed that there was no change in the percentage of the administered dose remaining in the liver after 5 hr in either of the experimental groups (Table 2). There was no change in the amount of vitamin K_1, vitamin K_1 2,3-epoxide or in their ratio in the phenobarbitone-induced rats compared with the appropriate saline-treated controls. The radioactivity not ether-extractable contained as yet unidentified, metabolites more polar than vitamin K_1 2,3-epoxide.

DISCUSSION

Vitamin K_1 is essential for normal blood coagulation because it is a cofactor for the post-ribosomal synthesis of clotting factors II (prothrombin), VII, IX and X [12]. The vitamin K_1-dependent step in clotting factor synthesis involves the γ-carboxylation of glutamic acid residues in these clotting factor precursors [13]. During the γ-carboxylation process, vitamin K_1 is converted into an inactive metabolite, vitamin K_1 2,3-epoxide, which either undergoes reduction back to the vitamin by vitamin K_1 epoxide reductase or is metabolized further to, as yet, unidentified metabolites [14]. The interconversion of the vitamin and its 2,3-epoxide occurs primarily in the rough endoplasmic reticulum of hepatocytes and is referred to as the vitamin K_1–K_1 epoxide cycle [15]. However, the major route of vitamin K_1 metabolism involves ω-oxidation and β-oxidation in the mitochondria followed by glucuronidation in the endoplasmic reticulum [16, 17]. Only when coumarin anticoagulants are administered does metabolism via the 2,3-epoxide become apparent [18]. The mechanism of action of coumarin anticoagulants is thought to be due to interruption of the vitamin K_1–K_1 epoxide cycle by inhibition of vitamin K_1 epoxide reductase [19].

In the present study we have shown that phenobarbitone enzyme induction increases the plasma clearance and decreases the terminal plasma half-life of vitamin K_1 when given in a pharmacological dose (1 mg/kg) to the rabbit. The terminal plasma half-life of vitamin K_1 determined for control animals (Table 1) is similar to that reported with a tracer dose (0.71 μg/kg) of vitamin K_1 [4, 10] indicating that clearance from plasma is not dose-dependent. However, the pharmacological response to vitamin K_1, determined as the increase in plasma prothrombin complex activity in brodifacoum anticoagulated rabbits, was not altered by phenobarbitone pretreatment indicating that the concentration of the vitamin at its physiological site of action in the liver is not affected.

To study further the effect of phenobarbitone on vitamin K_1 disposition, we monitored the excretion of vitamin K_1 metabolites in bile and the hepatic concentration of vitamin K_1 in the rat after administration of tracer and pharmacological doses of the vitamin. During 5 hr after intravenous administration of a radio-tracer dose of vitamin K_1, approximately 12% of the dose was excreted in the bile as water-soluble metabolites (Fig. 4). Similar results were observed with a pharmacological dose of the vitamin (Fig. 5) indicating that metabolism is not dose-dependent in the rat. Phenobarbitone enzyme induction significantly ($P \leq 0.05$) increased the excretion of biliary metabolites after both tracer and pharmacological doses of the vitamin. However, there was no corresponding decrease in hepatic vitamin K_1 concentrations at 5 hr (Table 2). The precise mechanism by which phenobarbitone enhances vitamin K_1 metabolism cannot be determined from the present data.

It is possible, however, that changes in liver blood flow or protein binding may be factors influencing vitamin K_1 pharmacokinetics or metabolism. Phenobarbitone, for instance, increases liver blood flow in the rat [7] which may alter a compound's pharmacokinetic profile. Enzyme induction also perturbs lipoprotein metabolism [29] which may lead to changes in protein binding of vitamin K_1 which is normally associated with plasma lipoproteins [21]. From the data presented above, however, the increase in biliary metabolites does not appear to reflect perturbation of the vitamin K_1–K_1 epoxide cycle as phenobarbitone did not affect the hepatic or plasma concentration ratio of vitamin K_1 2,3-epoxide: vitamin K_1 (Table 2). Furthermore, the plasma concentration ratio of vitamin K_1 2,3-epoxide:vitamin K_1 was similarly unaffected in the rabbit.

Taken collectively these data indicate that phenobarbitone enzyme induction enhances the metabolism of vitamin K_1 in the rat and rabbit without perturbing hepatic concentrations of the vitamin or the vitamin K_1–K_1 epoxide cycle. As liver concentrations are unaffected during phenobarbitone enzyme induction or long-term anticonvulsant therapy, this may explain why the offspring alone has a coagulant defect [1]. Vitamin K_1 is supplied to the foetus via the placenta which, in normal neonates, contains extremely low concentrations of the vitamin [22], and thus conditions which lower circulating maternal blood concentrations of vitamin K_1 may give rise to a coagulant defect in the offspring.

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